

SYMBIOTIC CHLOROPLASTS; THEIR PHOTOSYNTHETIC PRODUCTS AND CONTRIBUTION TO MUCUS SYNTHESIS IN TWO MARINE SLUGS

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Chloroplasts occur within the cells of the digestive diverticulum of some sacoglossan gastropod molluscs (Kawaguti and Yamasu, 1965; Taylor, 1967, 1968, 1970; Trench, 1969; Trench, Greene and Bystrom, 1969; Greene, 1969, 1970). These chloroplasts, derived from certain marine algae (Siphonales) produce oxygen in the light and fix much more ¹⁴C in the light than in the dark (Trench, 1969; Trench *et al.*, 1969; Greene, 1969; 1970; Taylor, 1971b).

It has been shown by radioautography that in the sacoglossans *Tridachia crispata* and *Tridachiella diomedea*, photosynthetically fixed ¹⁴C moves from the chloroplasts to various chloroplast-free animal organs, especially the pedal mucus gland. This gland secretes a carpet of mucus over which the slugs crawl (Trench, 1969; Trench *et al.*, 1969).

Recently, Trench, Trench and Muscatine (1970) showed that after specimens of *Placobranchus ianthobapsus* (Gould) were incubated in NaH¹⁴CO₃ in the light, the mucus secreted by the animals contained ¹⁴C-galactose and ¹⁴C-glucose. They suggested that chloroplast photosynthesis provided some of the substrates used by the animal in the synthesis of mucopolysaccharides.

To gain insight into the way sacoglossans utilize the products of chloroplast photosynthesis it is necessary first to identify these products in the animal hosts, and then to follow their incorporation into animal substrates. In addition, by comparing the products of symbiotic chloroplasts in their animal hosts with those of intact siphonous algae, possible modification in chloroplast function in the animal cell milieu may be detected.

This paper compares the products of photosynthetic ¹⁴C fixation by chloroplasts in the slugs *Tridachia crispata* (Mörch) and *Tridachiella diomedea* (Bergh) with those of chloroplasts in the siphonous marine alga *Caulerpa sertularioides*. The incorporation of chloroplast products into animal mucus is described in detail and the turnover of ¹⁴C in the pedal mucus gland of *Tridachia* is estimated.

MATERIALS AND METHODS

(1.) *Collection and maintenance of specimens*

Tridachia crispata and *Caulerpa sertularioides* were collected from the reefs off Port Royal and Discovery Bay, Jamaica, kept in the shade (about 900-1200 foot candles) in plastic tanks and used within a few days of collection.

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Tridachiella diomedea was collected from mangrove lagoons in the vicinity of La Paz, Baja California, Mexico. Experiments were conducted on board the R. V. DOLPHIN the same day.

(2.) Experiments

(a) *Incubations in NaH¹⁴CO₃*. Whole animals and pieces of alga (2–3 cm) were separately incubated in sea water to which NaH¹⁴CO₃ was added to give initial activities of 10–25 μ c/ml. Specimens of *Tridachiella diomedea* were incubated in sunlight (1000–1500 foot candles in the shade) at a temperature of 21° C \pm 2° C.

Incubations of *Tridachia crispata* and *Caulerpa sertularioides* were carried out under ambient light conditions (900–1700 foot candles) or under constant intensity light conditions of 900 foot candles delivered by photoflood lamps. Temperature was maintained at 25° C \pm 1° C.

(b) *Incubations in ³H-leucine*. Whole specimens of *Tridachia crispata* were incubated under conditions similar to those described above for ¹⁴C in sea water to which ³H-leucine (100–250 mc/mm, Amersham, England) was added to give initial activities of 10–20 μ c/ml.

(3.) Analytical procedures

(a) *Chemical fractionation of ¹⁴C-compounds from animal and algal tissues after photosynthetic ¹⁴CO₂ fixation*. After incubation in NaH¹⁴CO₃, both plant and animal material were extracted under reflux in methanol-chloroform (1:2 v/v) for two hours. This fraction was then extracted with diethyl ether in a separatory funnel. Phase separation was effected by the addition of small quantities of 15% aqueous NaCl. The ether phase was washed with several small aliquots of distilled water, and 100 μ l was removed for counting. This extracted lipid fraction was deacylated and the products identified as described previously (Trench, 1971a, 1971b).

After withdrawing 100 μ l of the reconstituted aqueous methanol fraction for counting, the remainder was evaporated to dryness under reduced pressure at 40° C and radioactive compounds were recovered in absolute ethanol or dry pyridine. This fraction is referred to as the "intermediary metabolites" fraction. The efficiency of recovery of ¹⁴C was 85–95%.

Tissues insoluble in methanol-chloroform were hydrolyzed in 3 M KOH in 80% ethanol. On neutralizing the solution, the precipitate formed was collected by centrifugation. An aliquot of 100 μ l of the supernatant, the "protein" fraction, was withdrawn for counting, and the remainder was stored for subsequent analysis.

The alkali insoluble precipitate was treated with 1 M HCl at 100° C for three hours. When neutralized, all the ¹⁴C in this fraction from animal tissues was reprecipitated. By contrast, algal tissues were completely solubilized by this treatment. An aliquot of 100 μ l of algal tissue hydrolysate ("polysaccharide" fraction) was removed for counting. The remainder was stored for subsequent analysis.

The 1 M HCl insoluble fraction from animal tissues was hydrolyzed in 3 M HCl for 4 hours at 100° C. The solution was neutralized and yielded an insoluble substance which did not dissolve in hot 5% TCA or hot formic acid. This fraction which contained less than 3% of the total fixed ¹⁴C was not analyzed further. The

insoluble substance was removed by centrifugation and an aliquot of 100 μ l of the supernatant (animal "polysaccharide") was collected for counting. The remainder of the samples was stored for subsequent analysis by paper chromatography.

(b) *Paper chromatography*. Solvent systems used during this study were (i) phenol: water (100:40 w/v) for the first dimension and n-butanol: propionic acid: water (142:71:100 v/v) for the second dimension (Bassham and Calvin, 1957) for two dimensional chromatography on Whatman Nos. 1 and 4 papers; (ii) methyl-ethyl-ketone: pyridine: water: acetic acid (70:15:15:2 v/v), (von Holt and von Holt, 1968b); (iii) n-propanol: ethyl acetate: borated water (7:2:1 v/v) and (iv) pyridine: water: ethyl acetate: acetic acid (5:3:5:1 v/v), (Block, Durrum and Zweig, 1958); the latter three systems being used in one dimensional descending chromatography on Whatman No. 1 paper.

Radioactive compounds on developed chromatograms were located by radioautography with Kodak x-ray film and by automatic scanning of one dimensional chromatograms (Loughman and Martin, 1957). Unknown radioactive compounds were identified by "finger-printing" co-chromatography in at least two different solvent systems.

Sugars used in co-chromatography or those produced by hydrolysis of mucus were detected chemically with silver nitrate, p-anisidine hydrochloride or aniline phthalate; amino sugars by the Elson-Morgan reaction (Neuberger and Marshall, 1966).

(c) *Gas-liquid chromatography* (GLC). Samples of mucus secreted by *Tridachia crispata* were hydrolyzed in 3 M HCl for 4 hours at 100°C. The hydrolysates were evaporated to dryness and the trimethyl-silyl derivative produced by adding 0.2 ml bis (trimethyl silyl) acetamide to the dried sample and allowing it to react for 24 hours at 20° C. Carbohydrates were separated with 2% SE52 on Diatomite C in a Pye 104 dual FID gas chromatograph, coupled to a Servoscribe recorder. Peaks were identified by use of internal standards.

(d) *Collection of mucus*. Mucus was collected from animals by placing individuals in about 2 ml sea water in a beaker and gently swirling the beaker. This caused the secretion over a period of about 10–15 minutes of copious quantities of mucus. After removal of the animal the mucus was precipitated from solution by the addition of an equal volume of absolute ethanol. After cooling to 3° C, the mucus was sedimented by centrifugation.

(e) *Mucus hydrolysis*. Samples of mucus secreted by *Tridachia* and *Tridachiella* were hydrolyzed in 3 M KOH at 100° C for 4 hours, and the solution neutralized. The unhydrolyzed polysaccharide was reprecipitated by the addition of absolute ethanol to the neutralized solution at 3° C, and collected by centrifugation. After several washes with cold 80% ethanol, the polysaccharide was hydrolyzed with 3 M HCl for 4 hours at 100° C. After hydrolysis, this solution was also neutralized. The products of hydrolysis were recovered in absolute ethanol or pyridine for analysis by paper chromatography.

Secreted ¹⁴C-mucus was precipitated as described above (3d) and hydrolyzed in sealed glass tubes in 3 M HCl at 100° C for 4 hours. The HCl was removed and the solutions were concentrated under vacuum prior to analysis by paper chromatography.

(f) *Estimation of total carbohydrate and sulphate in mucus*. The carbohydrate content of mucus was estimated using the phenol-sulphuric acid method of Dubois,

Gillies, Hamilton, Rebers and Smith (1956). Colorimetric measurements were made on a Bausch and Lomb Spectronic-20 spectrophotometer at 490 nm.

The sulphate content of mucus was determined by the method of Gibbons and Wolfrom (1962). Sedimented mucus was dried *in vacuo* over P_2O_5 to constant weight.

(g) *Measurement of radioactivity.* Radioactive samples were assayed on planchets with a thin end window Geiger-Müller counter. Corrections were made for self-absorption and background.

(h) *Tissue radioautography.* Animals incubated in $NaH^{14}CO_3$ or 3H -leucine were prepared for radioautography as previously described (Trench *et al.*, 1969). Paraffin-embedded tissues were sectioned at 5 μm , and were exposed to Kodak AR-10 stripping film at $-5^\circ C$ for 14 to 28 days. Radioautographs were developed as advised by the film manufacturer.

RESULTS

(1.) *Distribution of fixed ^{14}C in Tridachia, Tridachiella and Caulerpa*

After six hours photosynthesis in $NaH^{14}CO_3$ the distribution of ^{14}C in organic compounds in the two animals was very similar, while both were distinct from the pattern observed in the alga (Table I). Because of the relatively long incuba-

TABLE I
Percentage distribution of ^{14}C in Tridachia crispata, Tridachiella diomedea and Caulerpa sertularioides after six hours incubation in $NaH^{14}CO_3$ in the light. (10 $\mu c/ml$ $NaH^{14}CO_3$; 900-1000 foot candles)

	Lipid	Intermediary metabolites	Protein	Polysaccharide
<i>Tridachia crispata</i>	10 (8-12)	45 (41-47)	9 (9-10)	36 (34-43)
<i>Tridachiella diomedea</i>	12 (9-13)	40 (37-43)	8 (7-9)	40 (37-43)
<i>Caulerpa sertularioides</i>	28 (26-31)	10 (9-15)	10 (9-11)	52 (47-58)

Values represent averages of four experiments (range in parenthesis). Percentages in animals do not include ^{14}C in mucus secreted during the incubation period.

tion period, it was assumed that the pattern of distribution of ^{14}C in animal and algal tissues approached a "steady state" distribution. Further examination of the composition of these fractions was carried out by paper chromatography.

(a) *Lipids.* Lipids extracted from the animals yielded, after deacylation, many labelled compounds, among which occurred glycerol and traces of monogalactosyl-glycerol and digalactosyl-glycerol. These same glycolipids were also found after deacylation of lipids from *Caulerpa* but relatively much more ^{14}C was found in mono- and digalactosyl-glycerol (Table II). It seems reasonable to conclude from these comparisons that the glycolipids are characteristic of plant lipids (see also Thompson, 1965; Nichols, 1970) and that those detected in the animals were probably derived from symbiotic chloroplasts. If this interpretation is accurate, it would suggest that symbiotic chloroplasts only synthesize glycolipid to a limited extent.

(b) *Intermediary metabolites.* A wide range of small molecular weight compounds incorporated ^{14}C in the animals and in the alga, the majority of which were distributed amongst the sugar phosphates, amino and carboxylic acids (Table III). Little can be concluded from these data other than the fact that ^{14}C was found mostly in glutamic acid, alanine and succinic acids in both the animals and the plant. The extent to which this may represent dark fixation was not investigated, but dark fixation represents only 4–5% of photosynthetic fixation (Trench *et al.*, 1969). The remainder of the ^{14}C in this fraction was found principally in carbohydrates, as ^{14}C -galactose and ^{14}C -glucose in the animals and ^{14}C -sucrose and ^{14}C -glucose in the alga.

(c) *Protein.* Relatively small amounts of ^{14}C were detected in animal and plant protein (of Table I). Hydrolysis of animal protein yielded mostly ^{14}C -glutamic acid and ^{14}C -leucine. Algal protein hydrolysates were not analyzed.

(d) *Polysaccharide.* When this fraction of animal tissue was hydrolyzed in 3 M HCl, ^{14}C was found mainly in glucose, galactose, and an unidentified pentose in the ratios of 6:1:2 in *Tridachia* and 1:7:4 in *Tridachiella*. This fraction is of interest in that it may represent synthesized but unsecreted mucus. Hydrolysis of *Caulerpa* polysaccharide yielded ^{14}C -glucose, ^{14}C -galactose and ^{14}C -mannose in the ratio 1:5:3.

These results imply that the fate of photosynthetically fixed ^{14}C in the animals with symbiotic chloroplasts is distinct from that in the alga from which the

TABLE II

Percentage distribution of ^{14}C in products of deacylated lipids in Tridachia crispata, Tridachiella diomedea and Caulerpa sertularioides after photosynthetic ^{14}C fixation. Experimental conditions and numbers of replications as in Table I.

	Mono-galactosyl-glycerol	Digalactosyl-glycerol	Sulpholipid (?)	Glycerol	Unidentified and undeacylated lipid
<i>Tridachia crispata</i>	3	2	15	45	35
<i>Tridachiella diomedea</i>	5	2	10	43	35
<i>Caulerpa sertularioides</i>	16	19	7	3	55

chloroplasts are derived. This may indicate modification of function by chloroplasts in the animals as well as differences in the utilization of products of chloroplast photosynthesis by animal and plant tissues.

(2.) *Incorporation of products of chloroplast photosynthesis into animal mucus*

(a) *Analysis of mucus secreted by Tridachia and Tridachiella.* Under experimental conditions, green specimens of *Tridachia* containing chloroplasts secreted 300–400 $\mu\text{g/g}$ (fresh weight)/hr of mucus (expressed as glucose equivalents), while white animals (presumed to be chlorophyll free) secreted about 100–150 $\mu\text{g/g/hr}$.

Mucus from *Tridachia* contained 125–133 $\mu\text{g SO}_4\text{=}/\text{mg}$ dry weight of mucus, and that from *Tridachiella* contained 40–54 $\mu\text{g SO}_4\text{=}/\text{mg}$ dry weight mucus. It is not known whether sulphate is present as N-sulphate or O-sulphate or both.

Analysis of hydrolysates of mucus secreted by *Tridachia* by paper chromatography and GLC showed glucose, galactose, glucosamine, galactosamine and an unidentified pentose to be present, while hydrolysates of mucus secreted by *Tridachiella* contained glucose, galactose, possible traces of glucosamine and an unidentified pentose as detected by paper chromatography.

Alkaline hydrolysis of mucus from *Tridachia* and *Tridachiella* yielded a series of ninhydrin positive compounds. Although none of these were rigorously identified, compounds behaving chromatographically like leucine and glutamic acid were recognized. It is suggested that these amino acids were derived from the protein associated with the polysaccharide polymer, since it is known that many invertebrate mucins are associated with protein (Hunt, 1970).

(b) *Analysis of ^{14}C in secreted mucus.* Figure 1 shows the ^{14}C -labelled sugars in acid hydrolysates of mucus secreted by *Tridachia* and *Tridachiella* under similar experimental conditions. Most of the sugars detected in unlabelled mucus

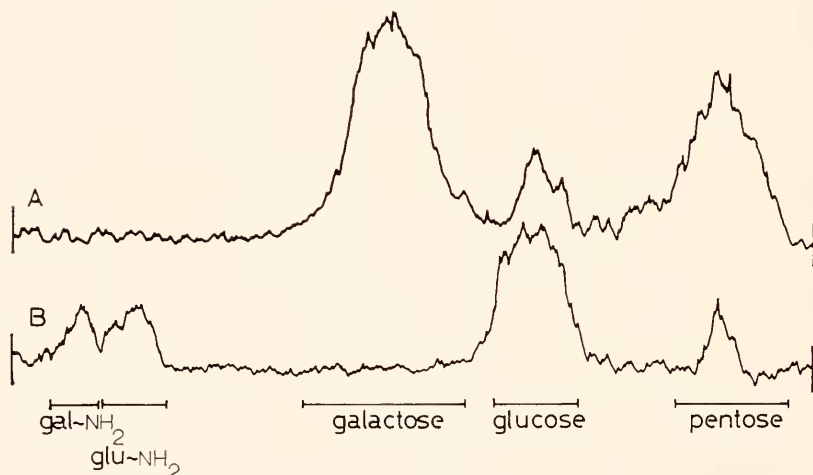


FIGURE 1. Chromatogram scans of ^{14}C -sugars produced by acid hydrolysis of ^{14}C -mucus secreted by *Tridachiella diomedea* (A) and *Tridachia crispata* (B) during incubation in $\text{NaH}^{14}\text{CO}_3$; solvent system, methyl-ethyl-ketone: pyridine: water: acetic acid, (70:15:15:2, v/v), 36 hours on Whatman No. 1 paper; gal- NH_2 , galactosamine; glu- NH_2 , glucosamine.

are represented, but the distribution of ^{14}C in these moieties is different in the two species of slugs (Table IV). In *Tridachia* most of the ^{14}C in mucus is in the glucose moiety, while in mucus from *Tridachiella*, galactose contained most of the ^{14}C , but a smaller amount of ^{14}C was also incorporated into glucose. Taking into account the data on ^{14}C fixed into carbohydrates (Table III) it is suggested that the labeled moieties detected in mucus were derived from chloroplast photosynthesis. Recalling from Tables III and IV that in *Tridachia* the major soluble labelled sugar is galactose, while the chief labelled component of mucus is glucose, it is tempting to suggest an interconversions of sugars before or during mucus synthesis. Such interconversions may have been less complete in *Tridachiella*, since galactose is the chief constituent of both the soluble sugar pool and the mucus sugar moieties. Either

TABLE III

Percentage distribution of ^{14}C in the "intermediary metabolites" fraction from *Tridachia crispata*, *Tridachiella diomedea* and *Caulerpa sertularioides* after photosynthetic ^{14}C fixation. Experimental conditions and numbers of replications as in Table I

	Glucose	Galactose	Sucrose	Sugar phosphates and amino acids	Carboxylic acids
<i>Tridachia crispata</i>	5	40	0	39	16
<i>Tridachiella diomedea</i>	9	27	0	48	16
<i>Caulerpa sertularioides</i>	13	0	29	30	28

The values given represent ^{14}C in the different compounds as estimated by scanning the developed radiochromatograms.

the two species of slugs have different modes of mucus synthesis, or the same mode which has been observed at different stages of a complex process.

(c) *Redistribution of ^{14}C in *Tridachia* after a pulse of $\text{NaH}^{14}\text{CO}_3$.* To obtain further information on the possibility of the interconversion of sugars in mucus synthesis, a series of pulse experiments were carried out on *Tridachia*. Specimens were incubated in $\text{NaH}^{14}\text{CO}_3$ in the light for 60 min and then transferred to unlabelled sea water for up to 24 hr. Animals were removed at 1, 3, 6, 9, 12 and 24 hr after the end of the pulse. At each sampling, mucus was collected from the sampled animal which was then killed in methanol-chloroform for subsequent chemical analysis.

Analysis of animals by chemical fractionation showed that immediately after the pulse, most of the ^{14}C in the "intermediary metabolites" fraction was in galactose. This is in agreement with the data in Table III. Over the next 24 hr there was a gradual decline in the relative amount of ^{14}C -galactose with a concomitant increase in ^{14}C -glucose, which reached a maximum shortly before the maximum specific activity of secreted ^{14}C -mucus was attained (Fig. 2).

These data strongly support the concept that in *Tridachia*, ^{14}C fixed by the chloroplasts is rapidly converted to galactose which is then available for conversion to glucose, presumably at or near the site of mucus synthesis. It is then possible to infer that in *Tridachiella*, less galactose is converted to glucose, and instead, galactose is incorporated directly into mucus.

(3.) *Rate of turnover of ^{14}C and ^3H -leucine in the pedal gland of *Tridachia**

(a) *Turnover of ^{14}C .* To gain information on the rate of turnover of chloroplast products in the pedal gland of *Tridachia*, specimens were incubated in $\text{NaH}^{14}\text{CO}_3$

TABLE IV

Percentage distribution of ^{14}C in mucus secreted by *Tridachia crispata* and *Tridachiella diomedea* during incubation in $\text{NaH}^{14}\text{CO}_3$ in the light

	Glucose	Glucosamine	Galactose	Galactosamine	5-C sugar
<i>Tridachia crispata</i>	53	24	0	12	11
<i>Tridachiella diomedea</i>	8	0	56	0	36

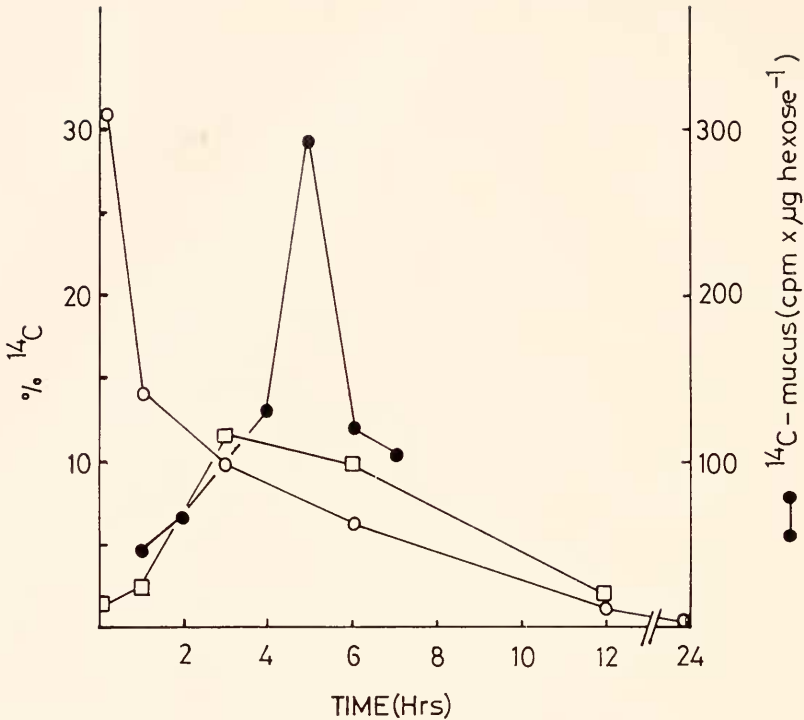


FIGURE 2. Relationship in *Tridachia crispata* between ^{14}C -galactose, ^{14}C -glucose and ^{14}C -mucus after one hour "pulse" label in $\text{NaH}^{14}\text{CO}_3$ in the light. Labeled glucose and galactose were extracted from the animals, while ^{14}C -mucus was collected from that secreted by the animals; ○—○, galactose; □—□, glucose; ●—●, secreted mucus.

in the light for 1 hr and then transferred to unlabelled sea water. Secreted mucus was collected at hourly intervals beginning immediately after removal of the specimen from the labelled medium, and the specific activity of the ^{14}C -mucus determined. Animals were also fixed in Bouin's fluid for subsequent analysis by radioautography.

Figure 3 shows that the specific activity of ^{14}C -mucus reaches a maximum at about 5 hr and then declines, suggesting a turnover time for ^{14}C of about 10 hours.

When the radioautographs were analyzed, an increasing density of silver grains was observed over the pedal gland up to about 6 hr after the pulse (see also Trench *et al.*, 1969), but very few grains were detected after 12 hr presumably because the labelled mucus had been secreted.

These observations are consistent with the estimate of a 10–12 hour turnover time for carbon in the mucus secreting pedal gland of *Tridachia* under these experimental conditions.

(b) *Turnover of ^3H -leucine.* In view of the observation that the polysaccharide secreted by *Tridachia crispata* had associated protein, it was of interest to ascertain whether exogenously supplied amino acids could be incorporated into mucus-secreting tissues. Specimens were therefore incubated in ^3H -leucine for 15

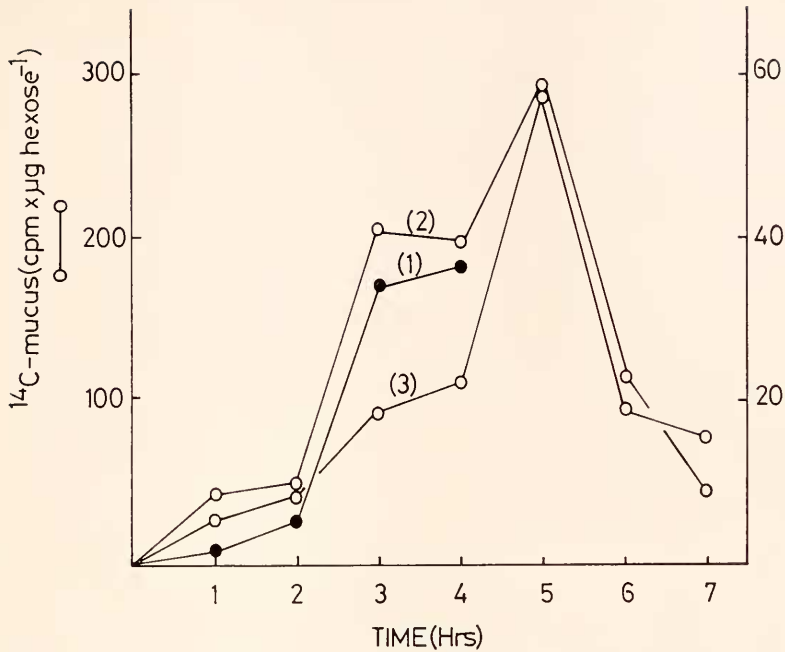


FIGURE 3. Specific activity of ¹⁴C-mucus secreted by *Tridachia crispata* after one hour pulse label in NaH¹⁴CO₃. Experiment (1) was conducted with ¹⁴C at a concentration of 10 µc/ml; experiments (2) and (3) at 50 µc/ml.

minutes, and then transferred to unlabeled sea water. Specimens were fixed in Bouin's fluid after 1, 3, 6 and 12 hours in the unlabelled sea water, and the location of radioactivity determined by radioautography.

After 15 minutes in ³H-leucine, most of the insoluble radioactivity in *Tridachia* was associated with the epidermis. After one hour in unlabelled sea water, radioactivity was detected in the end-bulbs of the tubules of the digestive diverticulum and in some (autochromatic) epidermal mucus glands. This contrasts with previous observations which showed that photosynthetic ¹⁴C from the chloroplasts was not incorporated into the epidermal mucus glands (Trench, unpublished). Metachromatic mucus cells did not incorporate high levels of radioactivity. Digestive tract tissue also incorporated ³H as did the pedal mucus gland.

After six hours most of the ³H was absent from the epidermal mucus cells, but ³H could still be demonstrated in the pedal mucus gland. After 12 hours however, the pedal gland contained only very little ³H, and the only remaining areas of relatively high activity were the end bulbs of the tubules of the digestive diverticulum.

These observations suggest (a) that the incorporation of amino acids into a polysaccharide-protein complex probably occurs in the pedal mucus gland of *Tridachia*, (b) that leucine may be an important constituent amino acid of the mucus secreted by the pedal mucus gland as well as the epidermal mucus and (c) that the rate of disappearance of ³H from the pedal gland after a pulse "pulse" incorporation is similar to that of ¹⁴C.

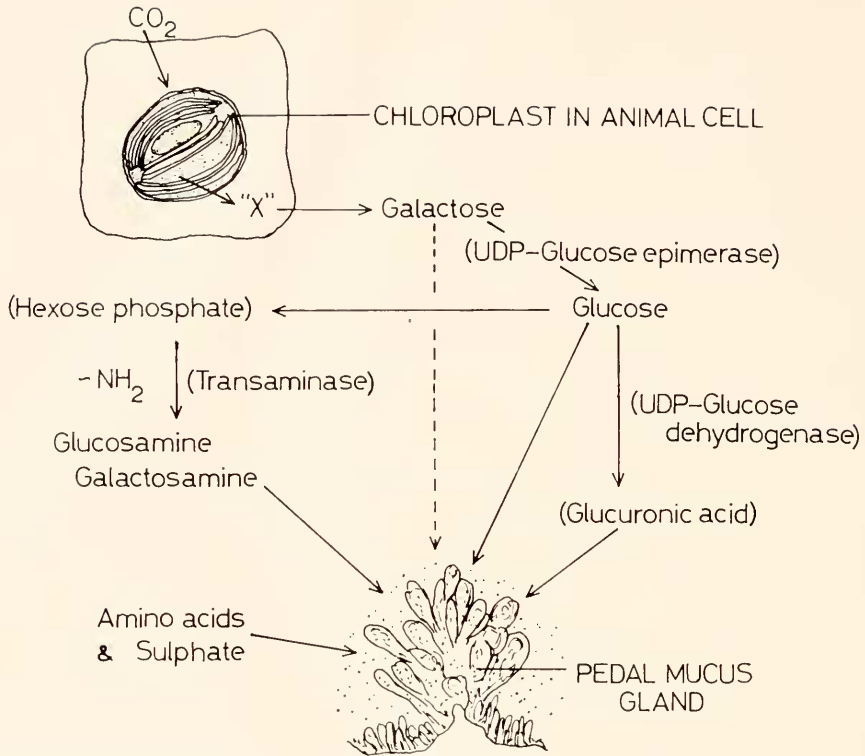


FIGURE 4. Schematic representation of a hypothetical pathway of carbohydrate moving between symbiotic chloroplasts and the pedal mucus gland of *Tridachia crispata*. Enzymes or intermediates in parenthesis have not been identified in the animals studied. Pathways indicated by dotted lines represent alternative possibilities as indicated by data from *Tridachiella* and *Placobranchus*.

DISCUSSION

The distribution of photosynthetically fixed ^{14}C in *Tridachia*, *Tridachiella* and *Caulerpa* shows marked differences. There could be two reasons for this observation. First, the metabolism of the chloroplasts could become modified in symbiosis and secondly, fixed ^{14}C released by symbiotic chloroplasts may be converted to animal products which are different from those of the plant.

Modification of chloroplast metabolism in animal hosts is suggested by the observation that, in the animals, only trace quantities of ^{14}C are detected in monogalactosyl and digalactosyl diglycerides, while in *Caulerpa*, a larger proportion of fixed ^{14}C incorporated into lipid is found in the galactolipids. This may indicate that less chloroplast membrane synthesis occurs in the slugs over a six hour period than in the plant. This interpretation would be consistent with the reduced chlorophyll synthesis by chloroplasts in slugs observed by Trench and Smith (1970) if the assumption that chloroplast growth and replication is directly correlated with chlorophyll and galactolipid synthesis is tenable (see Boasson and Laetsch, 1969; Constantopoulos, 1970).

By contrast, the difference in metabolism of ^{14}C -sugars between *Caulerpa* and the two slugs is more difficult to explain on the basis of modification of chloroplast behavior. Although ^{14}C -sucrose was found in the plant but not in the animals, it is not known whether this compound was synthesized in the plant cytoplasm or in the chloroplast. Previous studies have suggested that isolated chloroplasts synthesize sucrose (Tolbert and Zill, 1954; Gee, Joshi, Bils and Saltmann, 1965; Shephard, Levin and Bidwell, 1968; Bidwell, Levin and Shepard, 1970) while others maintain that the cytoplasmic fraction is essential (Walker, 1967). The absence of sucrose in the tissues of the slugs is consistent with the interpretation that sucrose synthetase is a cytoplasmic enzyme in the plant, but there is the possibility that the animal cells could convert sucrose released by the chloroplasts to animal sugars, or directly utilize the precursors of sucrose released by the symbiotic chloroplasts.

In the slugs, ^{14}C -galactose and ^{14}C -glucose were the major free sugars detected after fixation of $^{14}\text{CO}_2$; but whether these are located in the chloroplasts or in the animal tissues is unclear. The presence of free ^{14}C -glucose and ^{14}C -galactose in slugs with symbiotic chloroplasts after photosynthetic $^{14}\text{CO}_2$ fixation appears to be widespread. Greene and Muscatine (in press) also found these labelled sugars in ethanol extracts of the slugs *Elysia hedgepethi* and *Placobranchus ianthobapus* after 2.5 hours photosynthesis. The chloroplasts in these slugs are also derived from a siphonous alga. Comparative studies with chloroplasts isolated from the plant and from animals are currently being undertaken in the hope of clarifying the problem of distinguishing between substances synthesized and retained by the chloroplasts and those released and metabolized by the cell cytoplasmic enzyme systems.

The difference of incorporation of ^{14}C into insoluble compounds in the animals and the alga is also striking. Plant material was readily hydrolyzed in 1 M HCl, while animal material was more resistant. This may be a reflection of protective NH_2 groups in sugar amines in animal polysaccharides (Neuberger and Marshall, 1966). Acid hydrolysis of ^{14}C -labelled animal material yielded glucose, galactose and a pentose (similar to the products of secreted ^{14}C -mucus). These sugars were probably derived, at least in part, from mucus within the animal. Acid hydrolysis of ^{14}C -labelled plant insoluble material yielded glucose, galactose and mannose, which may have been derived in part from "cell wall" material, since *Caulerpa* is known to be rich in polysaccharides containing galactose and mannose (Percival and McDowell, 1967).

The incorporation of fixed ^{14}C into mucus is clear evidence that the animals can metabolize products released by the chloroplasts, but there is no data on how much carbon is provided by the chloroplasts for animal mucus synthesis. However, the observation that animals with chloroplasts secreted three to four times as much mucus as those without suggests that carbon supply from the chloroplasts is quantitatively important in mucus synthesis. The very rapid turnover of fixed ^{14}C in the mucus secreting pedal gland, demonstrated in the experiments, further supports this view.

The carbohydrate composition of mucus secreted by *Tridachia* and *Tridachiella* was generally similar. After hydrolysis, the sugars detected were glucose, glucosamine, galactose, galactosamine, and an unidentified pentose. Following incubations of the animals in $\text{NaH}^{14}\text{CO}_3$, photosynthetically fixed ^{14}C became chemically

incorporated into secreted mucus as ^{14}C -sugars. Thus, as previously suggested by Trench *et al.*, (1970) it is possible that one of the forms in which carbon moves from the chloroplasts to the animals is as sugar or sugar precursors (see also Smith, Muscatine and Lewis, 1969). The animals very likely possess the enzyme systems necessary for their interconversions. A hypothetical scheme depicting this is shown in Figure 4. Although the mode of transfer is not known, metabolites very likely enter the haemocoel and are distributed throughout the organism. The sites at which the interconversions may occur are also unknown, though from data available from other systems (see Huut, 1970), it is possible that an epimerase system may be located near the cells containing the chloroplasts, while other epimerases, transaminases and dehydrogenases may be associated with the mucus secreting cells themselves. Thus, the sugar amines and sugar acids may actually be formed in the Golgi apparatus of the cells that comprise the pedal gland (see Neutra and Leblond, 1966).

There is as yet no direct measurement of the proportion of fixed ^{14}C released by symbiotic chloroplasts. However, since 30% of the total ^{14}C fixed was recovered in mucus after 5 hr in the pulse label experiment (Fig. 2), and since mucus is obviously an animal product, then a minimum of 30% of the carbon fixed by the chloroplasts in *Tridachia* is released to the animal. It should be borne in mind however, that mucus synthesis is not the only fate of chloroplast photosynthetic ^{14}C , as indicated by the radioautographic studies of Trench *et al.*, (1969). Neither is there any available data on how much of the chloroplast products is used in respiratory pathways. With the data available, it is possible to make an estimate of the proportion of fixed ^{14}C released if the following three assumptions are valid; (i) that free galactose (in the "intermediary metabolites" fraction) is an animal product made from some unknown precursor released from the chloroplasts (see Fig. 4); (ii) that galactose itself is a precursor used in the synthesis of mucus, and (iii) that the chloroplasts do not themselves synthesize large quantities of ^{14}C -polysaccharide (unpublished results of preliminary experiments on isolated chloroplasts suggest that this may in fact be the case). From these assumptions, it is possible to calculate from the data in Tables I and III that after 6 hr photosynthesis in $\text{H}^{14}\text{CO}_3^-$, 51%–54% of the total ^{14}C fixed in *Tridachia* and *Tridachiella* can be found in free galactose and mucus. It is therefore suggested that at least half of the carbon fixed photosynthetically by symbiotic chloroplasts is released to the tissues of the host animal.

The detection of free sugars and amino sugars in hydrolysates of mucus does not provide proof that these occur as the unsubstituted hexoses in native mucus. For example, N-sulphate, O-sulphate or N-acetyl groups would have been removed during acid hydrolysis. Similarly, the presence of a pentose in hydrolysates of mucus could be an indication of the occurrence of uronic acid in the native mucus, since under conditions of acid hydrolysis, uronic acids may be decarboxylated (Brimacombe and Webber, 1964).

Similar to the mucus secreted by *Placobranchus* (Trench *et al.*, 1970) mucus secreted by *Tridachia* and *Tridachiella* appears to contain a protein moiety associated with the polysaccharide polymer. The observations on the incorporation of ^3H -leucine into mucus-secreting tissues by *Tridachia* suggest the existence of a polysaccharide-protein complex. Most studies of mucus from molluscs have reported the presence of protein (Brimacombe and Webber, 1964; Masamune and

Yosozawa, 1965), and that from *Tridachia* and *Tridachiella* appears to conform to the pattern. Although no details of the structure of mucus from the pedal gland of *Tridachia* and *Tridachiella* can be presented, it is very likely a sulphated acid polysaccharide associated with protein.

It is important to stress that the experiments described represent studies of intact systems containing chloroplasts, and not the isolated chloroplasts themselves. To determine the fate of fixed ^{14}C in the plant and animal systems, it would be advantageous to show the form in which ^{14}C is released from the chloroplasts in animal and plant cells. Experiments designed to study these problems are now in progress.

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SUMMARY

1. The products of photosynthetic $^{14}\text{CO}_2$ fixation by symbiotic chloroplasts in the marine sacoglossan opisthobranch gastropods *Tridachia cristata* (Mörch) and *Tridachiella diomedea* (Bergh) were compared with those of the siphonous green alga *Caulerpa sertularioides*.

2. After six hours photosynthesis in $\text{NaH}^{14}\text{CO}_3$, the distribution of ^{14}C in organic compounds in the two slugs was similar, but different from that in the plant.

3. The major soluble ^{14}C -labelled carbohydrates found were glucose and sucrose in the plant and glucose and galactose in the slugs.

4. The plant contained appreciable ^{14}C -galactolipids, while little ^{14}C could be detected in galactolipids in chloroplasts in the animals. In both slugs and alga, large quantities of ^{14}C were incorporated into polysaccharide.

5. After pulse-labelling for one hour, ^{14}C -galactose was the major soluble carbohydrate detected in *Tridachia*. Subsequently, the galactose decreased with a concomitant increase in ^{14}C -glucose. Thereafter maximum ^{14}C -glucose was detected in secreted ^{14}C -mucus suggesting utilization of galactose and glucose in mucus synthesis.

6. It is estimated that the rate of turnover of ^{14}C in the mucus-secreting pedal gland of *Tridachia* is 10–12 hours, suggesting that chloroplast products play an important role in animal mucus synthesis.

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